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Effects on the Mitosis of Normal and Tumor Cells Induced by Light Treatment of Different Wavelengths

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Objective: **Although the background of laser therapy by means of low level energy and power is still only partially understood, there are nevertheless promising reports from clinical studies concerning pain treatment, the acceleration of wound healing, and the modulation of cell functions. In order to contribute to the understanding of such a phototherapeutic procedure cell experiments were performed.**
Materials and Methods: **The influence of light (λ = 410, 488, 630, 635, 640, 805, and 1,064 nm and broad band white light) on the proliferation of cells was investigated on skeletal myotubes (C2), normal urothelial cells (HCV29), human squamous carcinoma cells of the gingival mucosa (ZMK1), urothelial carcinoma cells (J82), glioblastoma cells (U373MG), and mamma adenocarcinoma cells (MCF7) in a computer-controlled light treatment chamber. The cellular response was tested by way of the following methods: The rate of mitosis was determined by counting the single cells after Orcein-staining. The proliferation index measurements were based on the BrdU incorporation during the DNA synthesis. Statistics were performed using unpaired Student's t-test procedures, stating $P < 0.05$ to be significant and $P > 0.05$ not to be significant.**
Results: **Twenty-four hours after light treatment, a significant increase in the mitotic rate of J82 and HCV29 cells was determined when illuminated with λ = 410 nm, λ = 635 nm and λ = 805 nm, respectively. C2 cells showed an increase only after λ = 635 nm illumination. In all three cell lines, a maximum mitotic rate was determined after an irradiation between 4 and 8 J/cm^2, while a reduced mitotic rate was measured at 20 J/cm^2. MCF7, U373MG, and ZMK1 cells showed a slight decrease in the mitotic rate with increasing irradiation independent of the wavelength used. When an irradiation of 20 J/cm^2 was applied, all cell lines showed a slight decrease compared to the controls independent to the wavelength used. White light as well as λ = 1,064 nm does not affect the mitotic rate in this irradiation range. No significant differences in the effects could be determined when the irradiance changed between 10 and 150 mW/cm^2 at certain irradiation values. The BrdU test did not show any significant al-**

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terations with respect to possible light induced processes compared to the controls.
Conclusions: **Dependent upon the irradiation parameter, light of a defined wavelength does affect the mitotic rate of both normal as well as tumor cells. It could be hypothesized that the action spectra of the cellular response indicate the participation of endogenous porphyrins and cytochroms as primary photoreceptors. Taking into account all light induced processes, the term biomodulation should preferably be used. Lasers Surg. Med. 25:263–271, 1999.**

Key words: action spectra; biostimulation; biomodulation; low level laser therapy; wound healing

INTRODUCTION

Low doses of visible light, often delivered from laser light sources, have been used in clinical and preclinical studies for nearly 30 years to modulate cell function, lessen pain, and accelerate healing of soft tissue injuries [1]. Photoillumination at low energy levels is reported to generate significant bio-effects in biochemical, physiological, and proliferate phenomena in various organic components [2]. The light dose dependency (irradiation and irradiance) of biomodulative effects induced by continuous wave and pulsed laser light with different wavelengths and different cellular systems has already been investigated [3–6]. Also, the adhesion capability after light-induced biomodulation was studied to investigate processes associated to wound healing [5]. Laser irradiation with certain parameters was said to affect oxidative processes in cells. It was therefore suggested that laser light could activate redox reactions in the respiratory chain by exciting mitochondrial components [3,5,6] and membrane components [3]. This could lead to changes in intracellular ion concentrations, which play a vital role in cell proliferation [3,6] and/or this could stimulate the generation of reactive oxygen species [3,5,6]. Although numerous light-induced alterations and responses have been found on the cellular level, which may have considerable biological and clinical importance, the primary photo-acceptor has still not been identified. The biomodulating action of visible and near-infrared light is due to the excitation of endogenous chromophores. A comparison of the action spectrum of the cells to the absorption spectrum of potential photo-absorbers showed similarities to chromophores such as flavins, cytochromes of the respiratory chain, mitochondrial enzymes, and porphyrins, which are suggested to be the primary photo-target [3,5–8]. In clinical practice, a total irradiation of about 5 J/cm^2 at irradiances of up to 200 mW/cm^2 was delivered [1]. This kind of laser therapy was, therefore, named "low intensity," "low level," or "low power" therapy to emphasize the non-thermal character of the treatment.

To contribute to the understanding of the potential impact of the biomodulative phototherapeutic technique and to elucidate the underlying (photo)biological mechanisms, the proliferation of cells of different origins and different grades of malignancy was investigated and compared to normal cell lines. The experiments performed took into account the wavelength dependency of the effect leading to the action spectra of the different cell lines. Furthermore, the variation of irradiation and irradiance will show the optimum laser treatment parameters and thus to support the terms low power/energy/level therapy. Finally, the dependency of the measured effect to coherent and non-coherent light will be investigated.

MATERIALS AND METHODS

Cells

The effects of light were investigated on five human (epithelial like morphology) and one murine (myoblast like morphology) cell lines. While the J82 cell line associates from a poorly differentiated, invasive, stage T3 transitional cell carcinoma of the human bladder [9], the HCV29 cell line derives from a transformed but non-malignant bladder epithelium, which lacks bladder tumor-specific antigen [10]. The MCF7 cell line is derived from pleural effusion of a patient with metastatic breast cancer (estrogen-receptor-positive) classified as an adenocarcinoma of the mammary gland [11–13] (ATTC-catalog: HTB22; number 30, Deutsches Krebsforschungszentrum, Heidelberg, FRG). The U373MG cell line (ATTC-catalog: HTB17; number 42, Deutsches Krebsforschungszentrum, Heidelberg, FRG) is derived

from a glioblastoma-astrocytoma of the human brain and the ZMK1 associates from a well-differentiated (G2) squamous cell carcinoma (stage IV, T4N2M0) of the gingival mucosa [11,12,14]. The C2 myogenic cell line derives from the thigh muscle of normal C3H mice (ATTC-catalog: CRL1772) [15].

All cells were grown in RPMI 1640 (Biochrom, Berlin, FRG) and supplemented with 10% fetal bovine serum (FBS). Cells were maintained under standard conditions (T = 37°C, humidified atmosphere 5% CO_2/95% air) as subconfluent monolayers and were subcultivated twice a week. Exponentially proliferating cells were harvested by means of 0.05% trypsin/0.02% EDTA (GIBCO/BRL) and resuspended in fresh medium. Twenty-four hours before light treatment, the cells were seeded into six-well plates (NUNC, Wiesbaden, FRG) at a concentration of 30,000 cells for non-urothelial cells (C2, MCF7, U373MG, and ZMK1) and 50,000 cells for urothelial cells (HCV29 and J82) per well. A minimum of n = 6 wells was evaluated for each parameter tested in the mitotic index experiments by single cell counting in independent experimental attempts. DNA-synthesis experiments were performed in 96-well plates (NUNC, Wiesbaden, FRG) and 18–24 wells were evaluated for each parameter.

The time dependency of the biomodulative effect was investigated only on C2 cells, which were synchronized by means of withdrawing the serum. Therefore, at the time of seeding into the microtiterplates the cells were in the G1-phase [16].

Experimental Set-Up

Light of different wavelength was fed via a flexible fiber to a specially designed light treatment chamber, which enabled computer-controlled reproducible irradiation [17]. A speckle pattern on the irradiation area disturbed a homogenous light intensity distribution. Therefore, the fiber next to the fixed fiber end was shaken at a high frequency, which caused movements of the speckle pattern. The result was that the intensity distribution of the speckle pattern was averaged. It could be observed, during occurrence of the speckle pattern, that the coherence of the laser light was not influenced by the multimode fiber used. Consequently, the light intensity distribution on the irradiated area showed maximum variations of ±10%. In addition, the irradiation chamber was kept at a constant temperature (T = 37 ± 0.5°C).

The irradiance was measured by means of a calibrated powermeter before and after each light treatment and was monitored during illumination. The irradiation varied between 0 J/cm^2 and 20 J/cm^2. In the case of C2 and ZMK1 cells, the dependency of the irradiance was investigated using the parameters 50 mW/cm^2 and 150 mW/cm^2. HCV29 and J82 cells were illuminated with irradiances of 10 mW/cm^2 and 100 mW/cm^2. The experiments to get the action spectrum of the cellular response were performed at an irradiance of 10 mW/cm^2 for each cell line.

The following lasers lines were used to investigate the wavelength dependency of cellular responses: λ = 410 nm (Kr^+-laser); λ = 488 nm (Ar^+-laser); λ = 630 nm, 635 nm, and 640 nm (Ar^+-pumped tunable dye laser); λ = 805 nm (GaAlAs-diode-laser, FWHM = 25 nm); and λ = 1,064 nm (Nd:YAG laser). Additionally, a non-coherent Xe-short-arc lamp of an endoscopic white light source emitting at λ = 380–750 nm was used.

MEASUREMENTS

Two different methods were used to determine the biomodulative effect of light-induced processes. Independent experimental procedures were carried out for each of the methods. The first test method measured the proliferation of the cells (C2, HCV29, J82, and ZMK1) based on the BrdU incorporation during the DNA synthesis by means of the BrdU-test (Test-Kit, Boehringer, FRG) and ELISA reader technique starting 0, 3, and 24 hours after irradiation. The preparation of the BrdU-test took 3–4 hours inclusive of the 2 hours incubation with BrdU. The second test method used determined the rate of mitosis through Orcein-staining of the chromosomes [18] 20 to 24 hours after light treatment of the cells (C2, HCV29, J82, MCF7, U373MG, and ZMK1). An additional approach determined the rate of mitosis of synchronized C2 cells at 3, 6, 18, and 24 hours after light treatment. Controls without light treatment were made of each microtiterplate. With regard to reproducibility and statistics, each series of experiments was repeated for at least six times in independent experimental approaches.

The autofluorescence emission spectra of cell pellets (HCV29, J82, and U373MG) were measured with the help of an optical multichannel analyzer system (Stemmer Imaging, Germering,

FRG) during excitation with a Kr^+-laser emitting light of $\lambda = 410$ nm.

Evaluation

After Orcein staining, the mitotic cells (prophase and telophase) in the culture plates were counted by means of an inverted microscope (magnification ×200) 20–24 hours after illumination. Each single well was counted twice by two independent persons, blinded to each other's findings as well as to the light treatment parameters. Each person evaluated each single well, respectively, and each single attempt of each parameter under investigation. In order to receive a total number of about n(cell) = 1,000 unstained cells, about six to ten fields of visions per single well were evaluated. The number of unstained cells was added up. At the same time, the stained cells n(mito) were counted in each field of vision and were added up, too. For each single well, a relative value RV of mitotic cells, defined by RV = n(cell)/n(mitose), was determined. The RV-values of the two controls of each microtiterplate were averaged. The rate of mitosis, defined by MR = RV(test)/$RV(control)_{mean}$, was determined by the RV of the test – groups in relation to the mean value of the controls $RV(control)_{mean}$. The BrdU-staining procedure was measured by means of the standard ELISA reader technique (Emax, MWG Biotech, Ebersberg, FRG). The results were then compared to the controls of the same microtiterplate.

Data of the same experimental parameter was averaged and the standard deviations were calculated. Taking into account the wavelength of laser treatment, these were plotted versus the applied irradiation. As a result, the controls were determined to the value 1 indicating 100%. A value of 0.8 therefore indicated an inhibition of 20%. Statistics were performed using unpaired Student's t-test procedures, stating $P < 0.05$ to be significant and $P > 0.05$ not to be significant.

Finally, action spectra of the cellular response determined by means of the mitotic rate were created. To do so, only that data of the mitotic rate was used which was induced by light parameters at an irradiation of 4 J/cm^2 and an irradiance of 10 mW/cm^2 for each cell line used.

RESULTS

The fluorescence emission spectra were analyzed in order to identify emission bands of well-defined endogenous fluorophores (e.g., porphyrins, NADH, flavins, etc.). A comparison of the spectra prior to and after illumination showed neither cell-specific fluorescence emission nor alterations in the autofluorescence emission. Therefore, a spectroscopic identification of light-induced cellular changes was not possible.

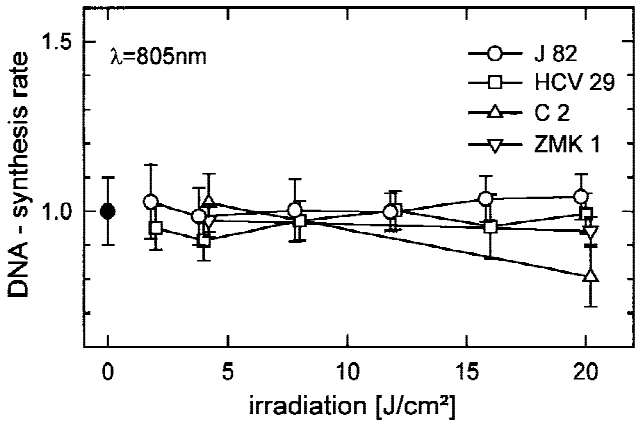

Fig. 1. The DNA synthesis rate (mean, standard deviation) does not significantly depend on the applied irradiation 24 hours after light treatment. Furthermore, no significant differences could be determined 0 and 3 hours (data not shown) after 805 nm-laser-illumination as well as between the cell lines under investigation. Controls were marked at 0 J/cm^2 (closed circle).

As shown in Figure 1, the DNA synthesis rate seems to be independent of the applied irradiation 24 hours after treatment with $\lambda = 805$ nm. The DNA synthesis rate does not differ significantly from controls indicated at 0 J/cm^2. The determination of the DNA synthesis rate by means of the BrdU-test immediately (t = 0 hours) and 3 hours after light treatment showed the same results. These indicated that a treatment of the cells with $\lambda = 805$ nm had no influence on the DNA synthesis rate when determined by the BrdU-test (Fig. 1).

Synchronized C2 cells showed stimulating as well as inhibitory effects on the mitotic rate 6, 18, and 24 hours after illumination with $\lambda = 805$ nm. In Figure 2, the stimulating effect due to an irradiation of 4J/cm^2, as well as a decreased mitotic rate after 20 J/cm^2 irradiation, is shown in comparison to the controls. The mean values measured at 18 and 24 hours after the treatment differed from the controls. But only the stimulating illumination with irradiation of 4 J/cm^2 could be determined to be significant with respect to the controls carried out 24 hours after treatment.

The mitotic rate of non-synchronized cells 24 hours after light treatment is summarized in Figure 3. While C2, HCV29, and J82 showed a sig-

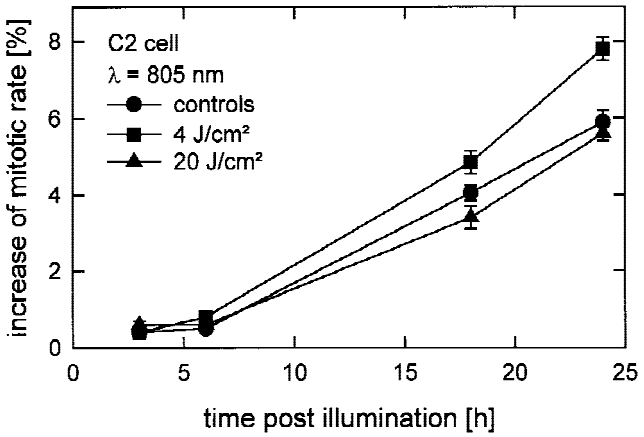

Fig. 2. The mitotic rate (mean, standard deviation) of synchronized C2 myotube cells plotted versus post-light treatment time measured by means of Orcein-staining 3, 6, 18, and 24 hours post illumination. Significant differences to the controls could be determined 18 hours after illumination and for 4 J/cm² 24 hours after illumination.

nificant increase of the mitotic rate after an irradiation between 4 and 8 J/cm² of λ = 410 nm, λ = 630 nm, λ = 635 nm, λ = 640 nm, and λ = 805 nm, the wavelengths of λ = 488 nm, λ = 1,064 nm, and white light did not result in any significant changes in comparison to the controls mentioned at 0 J/cm². The mitotic rate decreased with higher irradiation. The MCF7, U373MG, and ZMK1 cells responded with a decrease in the mitotic rate when exposed to light with 2–8 J/cm². They then remained stable up to 20 J/cm² when using λ = 630 nm, λ = 635 nm, and λ = 805 nm laser light.

The dependency of the mitotic rate of J82 cells for irradiations of up to 100 J/cm² for different wavelengths was tested, too. In contrast to controls, a stimulating effect of λ = 635 nm and λ = 805 nm laser light could be determined which persisted to an applied irradiation of 50 J/cm². At an irradiation of more than 70 J/cm², the photo-induced effect resulted in a mitotic index not significantly different to the controls (data not shown).

Although the results indicated that the mitotic rate was dependent on the irradiation applied, no change in the mitotic rate could be determined in dependency of the irradiance. This could be observed when the biomodulated effects on C2 and ZMK1 cells at irradiances of 50 mW/cm² and 150 mW/cm² were compared. The same was valid when HCV29 and J82 cells were illuminated at 10 mW/cm² and 100 mW/cm² (data not shown).

The cell line-specific and wavelength dependent responses in terms of the mitotic rate induced by light application of an irradiation of 4 J/cm² at an irradiance of 10 mW/cm² for each cell line is shown in Figure 4. The wavelength dependent inhibition, stimulation and stagnation reflects the action spectra of the initial photo-absorber. Three main peaks of increased mitotic rates could be observed at λ = 410 nm, λ = 635 nm, and λ = 805 nm. Coherent laser light of λ = 1,064 nm and non-coherent broad band white light did not show any effect on the mitotic rate.

DISCUSSION

Phototherapeutic treatments involving low levels of coherent or non-coherent light are still a controversial topic within the medical community [1] and there is evidence that published studies are often poorly controlled and incomplete. Several studies seem to provide clear evidence that this kind of treatment alters animal and bacterial cellular processes by means of non-thermal powers in a wavelength-dependent manner [3–6,19–21]. However, the mechanisms of the interaction between low levels of visible light and cells or tissues is not fully understood [1]. It has been claimed that mitochondrial respiratory chain components exhibit wavelength-dependent action spectra, hence the respiratory chain could be the starting point for any effects induced by small light doses [3,5,6,20].

The results obtained refer to the cell-specific responses to light treatment and to the problems of identifying the initial photo-acceptor. The action spectra in Figure 4 show the main cellular response in the wavelength ranges of about λ = 410 nm, λ = 635 nm, and λ = 805 nm. These findings correlate with the reported proliferation of fibroblasts biomodulated with light of λ = 633 nm and λ = 780 nm [3]. In this case, endogenous porphyrins and cytochromes as well as biopolymers were suggested to represent the primary photo-absorber. Results on the biomodulation in cell adhesion or RNA and DNA production lead to the assumption, due to the determined action spectra, that oxidized and reduced cytochrome a/a3 might represent the primary light-absorbing molecule [5,6]. Unfortunately, simple fluorescence measurement techniques did not seem sensitive enough to detect a fluorescence emission to clarify this question. It was found that the action spectra of visible and near-infrared light of various growth-stimulating and damaging effects of

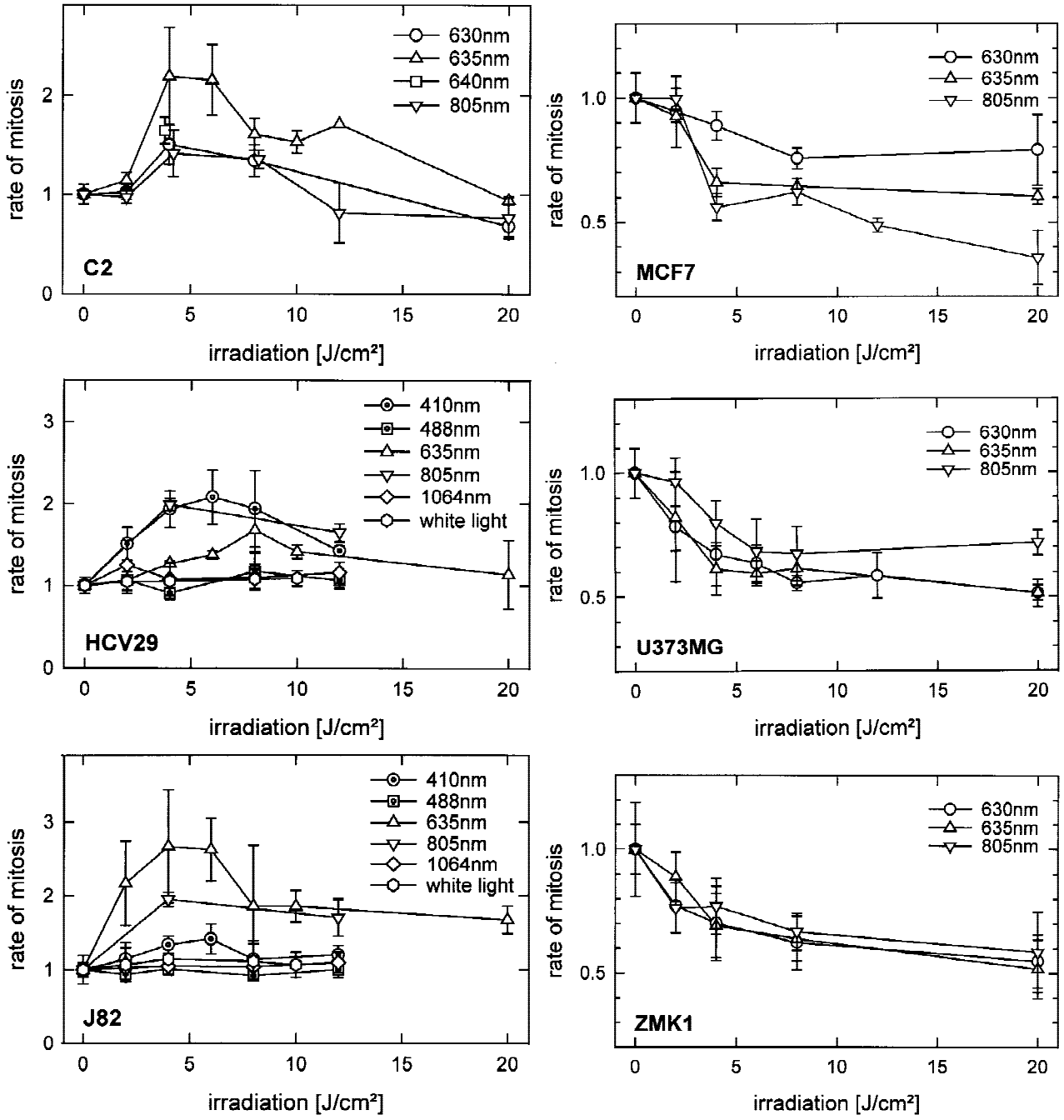

Fig. 3. The responses to light treatment is cell line specific [C2 (top left); HCV29 (middle left); J82 (bottom left); MCF7 (top right); U373MG (middle right); ZMK1 (bottom right)]. For each cell line the mitotic rate (mean, standard deviation) depends on the applied irradiation and wavelength used 24 hours post illumination. Controls were plotted at 0 J/cm^2.

certain organisms correspond to the absorption bands of respiratory chain components such as the flavins and the cytochromes [3–5,22,23]. It was reported that the absorption of light in this spectral range resulted in an increase of the intracellular pH which stimulated the mitosis [24]. Support in favor of the assumption that the primary photo-acceptors are pigments located in the mitochondrion is provided by the observation of enhanced ATP-synthesis in isolated mitochondria at appropriate wavelengths [7,24–26]. As the spectrum [5–7,27] of cytochrome a/a3 or cytochrome oxidase extends with a weak tail to the near-infrared spectral range between $\lambda = 700$ nm

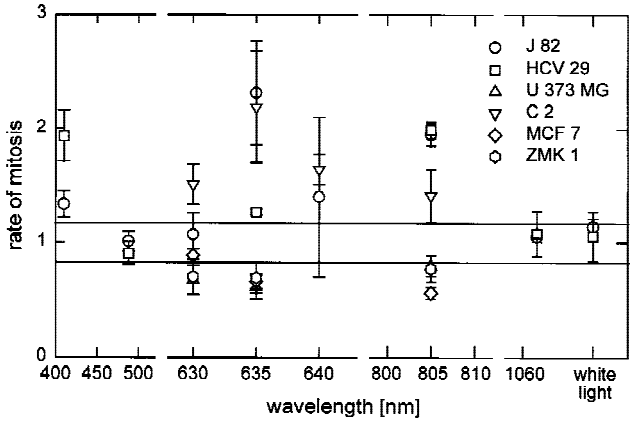

Fig. 4. Action spectra of the cellular response determined as the mitotic rate (mean, standard deviation) induced by light application of an irradiation of 4 J/cm^2 at an irradiance of 10 mW/cm^2. Note the breaks of the abscissa. The bar described the control value of the mitotic index is determined to be 1 and its interval of standard deviation.

and $\lambda = 900$ nm, these pigments seem to be the primary photo-acceptors of the experiments performed. With respect to the spectroscopic evidence that small concentrations of endogenous porphyrins [28] are among the primary photo-acceptors [7,8], these molecules could be helpful for the interpretation of the experiments due to their absorption spectrum in the visible spectral range between $\lambda = 400$ nm and $\lambda = 650$ nm [29,30]. Since biomodulation effects have a wide spectral range, it is assumed that there must be several different chromophores as photo-acceptor targets.

It was the aim of this study to look for cellular effects after low doses of light irradiation at different wavelengths. Our findings suggest that biomodulating effects can only be determined if sensitive detection methods are used. The integral measurement that determines the DNA synthesis with ELISA reader technique after BrdU staining showed no photo-induced alterations. The time-consuming and subjective method of single cell counting after Orcein staining seemed to be more appropriate. By means of this sensitive method it could be shown in the time dependency experiment that the mitotic rate of synchronized C2 cells differed significantly in comparison to the controls 24 hours after irradiation. With respect to this, the evaluation of all cell experiments was performed at this time after irradiation. The comparison of the mitotic rate of synchronized and non-synchronized C2 cells (Figs. 2, 3a) at identical irradiation parameters showed the same stimulation at 4 J/cm^2 and inhibition at 20 J/cm^2. Therefore it could be assumed that there is no special photosensitive phase within the cell cycle or that, at the time of irradiation, the cells were no longer in a synchronized growth, although being in the G1 phase at the time of seeding 24 hours before illumination [16].

So far, only a few tumor cell lines have been investigated with respect to biomodulation effects. Under these circumstances it must be considered critically whether a treatment modality showing stimulating as well as inhibitory effects on tumor cells should be developed. Further investigations under reproducible conditions on different tumor cell lines are needed to get detailed data on this problem.

Interestingly, both normal cell lines (HCV29 and C2) responded with stimulating effects at wavelengths corresponding to the absorption spectra of prophyrins and cytochromes, while at wavelengths with minor absorption of these absorbers a stagnation in the mitotic rate could be determined. The J82 tumor cell line showed a similar wavelength-dependent response. The tumor cells MCF7, U373MG, and ZMK1 showed inhibition. This might be interpreted that, on one hand, other absorbers are available or, on the other hand, the concentration and localization of absorbers differs to normal cells. Another interpretation lies in the origin of the cells, i.e., cells of the different organs may contain different photoactive components.

Looking at the background of biomodulation laser therapy, the response of cells in the neighborhood of a thermal or photodynamic laser treatment must be investigated. With respect to light dosimetry a certain zone exists, in which the illumination parameters of thermal or photodynamic laser treatment are comparable to biomodulation laser therapy. It could be hypothesized that the following biological effects occur within this zone. Tumor cells could respond with progress while neighboring normal cells could be down-regulated or vice versa, that is, normal cells within this zone respond with proliferation (e.g., wound healing) while neighboring tumor cells are destroyed.

Since there are several different procedures to deliver light to the area of interest [1], it appears important that considerations about light dosimetry should become of special interest. This study confirms that biomodulative effects occurred at irradiations of up to 20 J/cm^2. No significant changes in the induced effects could be observed when applying irradiances between 10 mW/cm^2 and 150 mW/cm^2. This kind of light

treatment is described to be non-thermal. The independence from the irradiance could be due to the fact that up to irradiances of 200 mW/cm^2 no significant increase of the temperature could be measured [31]. The reproducibility of the light dosimetry was guaranteed by means of the computer-controlled illumination chamber where each well was irradiated with intensity level variations of less than 10% [17]. The precise irradiation of the culture plates is a necessary prerequisite for the evaluation and comparison of such sensitive cell experiments.

To investigate the importance of the light coherence applied, HCV29 and J82 were irradiated using both laser light of $\lambda = 410$ nm and non-coherent Xe-short arc lamp filtered to emit light in the spectral range of $\lambda = 380$ nm to $\lambda = 420$ nm at an identical irradiance of 10 mW/cm^2. The comparison of the modulated mitotic rates within the irradiation range of 0 to 20 J/cm^2 showed identical responses. While HCV29 responded with an increased mitotic rate, J82 showed stagnation independent of the light source used {data not shown}. Apart from that, light treatment of HCV29 with broad band white light at irradiations of up to 100 J/cm^2 showed an increase in the mitotic rate. This may indicate that only the intensity of a certain wavelength or wavelength range is needed to induce biomodulative effects [32] and not the coherence of the light used.

Stimulation-irradiation therapy by means of low-dose X-ray-irradiation (0.2–0.5 Gy) [33,34] of benign diseases also showed effects, which could be described as biomodulation. In this case, the mechanisms are not completely understood either [33,34]. Compared to high-dose X-ray-irradiation of tumors the time schedules of the cell cycle in such a treatment were not taken into consideration. Different mechanisms (e.g., immunosuppression, inhibition of proliferation, anti-mitotic, anti-puriginous, and anti-inflammatory effects) are imaginable in the treatment of benign diseases in dermatology, degenerative diseases of the skeleton and acute inflammation.

So far, investigations on low-level visible light treatment have often been described as "biostimulation," which implies only stimulating but not inhibitory or stagnation effects. As shown by the results in the present paper, inhibitory effects also occur; hence the term "biomodulation" should be used since it takes into account all effects.

CONCLUSIONS

Low level light irradiation by means of coherent or non-coherent light results in a biomodulation of cellular activities in a wavelength dependent manner. Increased effects could be achieved using laser parameters such as irradiances in the range of 10 mW/cm^2 to 150 mW/cm^2 and irradiation between 4 and 8 J/cm^2. With respect to the volume where dosimetry parameters of this light treatment occurred in the depth of laser-treated tumors or malignancies, the cell-specific response had to be considered. It must be investigated in further experiments whether irradiances less than 10 mW/cm^2 can induce biomodulative effects. As the initial light-absorbing molecules are still unknown, the wavelength dependency effects [5,6,20,35] should be determined in greater detail by means of sensitive spectroscopic techniques. Furthermore, action spectra should be determined with respect to the cell lines and the measured response by using comparable irradiation parameters. With regard to the wide spectral range, where effects due to light treatment were observed, several different chromophores must be considered as photo-acceptor targets. However, it is likely that the photo-induced processes are just one aspect of the more general phenomenon of photo-signaling, which is getting more and more acknowledgment [36].

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